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Alfred R. Menino, Jr.

Immunocontraception targeting the zona pellucida shows promise as a safe, effective, and economically feasible method for controlling feral horse populations that roam public rangelands throughout the United States. The efficacy of porcine zona pellucida (pZP) vaccines to generate high titers can be evaluated with an indirect immunofluorescence assay or a sperm binding assay using equine, bovine or porcine oocytes and sperm. The objective of this research was to refine protocols for each of these assays that would yield high resolution results when comparing oocytes treated with high and low titer anti-pZP sera against various sets of control oocytes. Dilutions of rabbit anti-horse IgG-fluorescein isothiocyanate were varied in an indirect immunofluorescence assay which quantified fluorescence emitted from the zona pellucida. A dilution of 1:32 produced greater fluorescence intensity from oocytes treated with anti-pZP serum than control oocytes. Fluorescence intensity of oocytes

treated with the 1:32 dilution also varied significantly by the titer level, high or low, of anti-pZP serum used. A sperm binding inhibition assay was performed using a longer incubation time for sperm binding than had been previously documented. However, this modification did not produce any higher resolution than results reported in previous studies.

Key Words: equine, immunocontraception, bovine, oocyte

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Improving the efficacy of in vitro assays to detect physiologic differences in anti-porcine zona pellucida sera recovered from mares injected with SpayVac

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I understand that my thesis will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my thesis to any reader upon request.

Nicole Kristen Steigerwald, Author

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REVIEW OF THE LITERATURE

Wild Horse Population Problem

Public range lands in the United States are home to tens of thousands of feral horses and burros, believed to be descendants of Spanish horses brought to the New World in the 1500's, who interbred with released or escaped domestic horses. Through the 1900s, these animals were unprotected, and many were rounded up to be used as rodeo stock or slaughtered for meat. By the 1950's, there were fewer than 10,000 remaining, and public attention was brought to the diminishing numbers, as well as the inhumane methods used by private parties to gather, transport and slaughter these horses. Activists began to pressure the federal government to create laws protecting these animals, and in 1971 the Wild Free-Roaming Horse and Burro Act passed (BLM, 2012). It provided federal protection to herds on public lands, and charged the Secretary of the Interior with managing them. It is now a felony to remove these horses, harass, or kill them (BLM, 2006).

Without natural or human predators, however, feral horse populations can increase rapidly. On many rangeland areas they are at such high densities they cause damage to the ecosystem, compete with wildlife and cattle, and in extreme cases

experience severe food shortages themselves. In effort to control the population, the Bureau of Land Management (BLM, 2012) estimates the number of horses that can be supported on individual public rangelands throughout the country without causing excessive damage to the ecosystem, termed the Appropriate Management Level, and removes all animals in excess of this number. These animals are kept in holding areas while the BLM attempts to re-home them through the Adopt-A-Horse Program. Initially, BLM could not sell or destroy un-adoptable healthy animals. A 2004 amendment allowed for certain un-adoptable animals to be sold indiscriminately, however, BLM has not sold any of these to kill buyers for fear of public relations repercussions (BLM, 2012).

According to BLM (2012) there are currently 37,300 free roaming, feral horses, 11,000 in excess of BLM's Appropriate Management Level. There are an additional 46,500 animals in BLM's long term and short term holding pens. The number of horses adopted out is declining, and in the 2012 fiscal year, only 2,589 animals were adopted of the 8,285 gathered. The BLM is running out of holding capacity for these horses, as well as funds to pay for their care. It is clear another strategy is necessary to control these populations. Reproductive management to curb population growth appears to be the best solution (Garrot et al., 1991; 1992).

Methods of Reproductive Control

A wide variety of options have been studied as contraceptive methods for wild horses. However, the ideal contraceptive for wild horses must meet a number of

requirements, including long lasting and highly effective contraception from a single application, ability for remote delivery, safety for animals farther down in the food chain, and safety for target animals, including pregnant mares (Kirkpatrick and Turner, 1991).

Herd stallion sterilization had been suggested as a means of fertility control, however this would be ineffective and impractical for several reasons. Jannet et al. (2008) showed that vaccination against the reproductive hormone gonadotropin releasing hormone (GnRH) would be effective as contraception, but would cause suppression of mating behavior and secondary sex characteristics. Without secondary sex traits, dominant stallions would fail to keep their breeding bands together, and the social structure of feral horses would be disrupted (Kirkpatrick et al., 2011). Surgically vasectomizing stallions would cause infertility without behavioral changes, however this process carries the high risks and cost of capture, immobilization, and surgery. Furthermore, Asa (1999) found that herds with a vasectomized lead stallion will still experience 17% to 33% conception rates, due to sneak breedings by bachelor stallions. Instead, focus has been on finding a contraceptive method for mares.

Successful contraception has been observed using an intra-uterine device (IUD) in mares. Plotka et al. (1992) found that IUDs containing progesterone and ethinyloestradiol provided high contraception rates in feral mares over the three year study. However, it is necessary to use synthetic hormones because animals rapidly metabolize native hormones, such as progesterone and estradiol (Kirkpatrick et al, 1993). The possibility of these synthetic hormones being passed to animals farther down the food chain may make it difficult or impossible for these IUDs to be approved by the Food and Drug Administration and the Environmental Protection Agency (Kirkpatrick et al.,

1993). The cost of capture and immobilization also makes this method impractical compared to methods for which remote delivery is possible.

Immunocontraception is a more practical method for fertility control than hormonally based IUDs because it can be delivered remotely (Willis et al., 1994). Additionally, steroid hormones in the tissue of target animals could have a negative effect on predators, while proteins from an immunocontraceptive would be deactivated in the stomach of non-target animals should they be ingested orally (Warren et al., 1993). Immunocontraception causes infertility by introducing an antigen to trigger an active immune response in the target animal against some molecule necessary for reproduction (Kirkpatrick and Turner, 1991)

GnRH has been studied as a possible target for immunocontraception in mares. GnRH is a hypothalamic hormone that acts on the pars distalis of the pituitary gland to cause secretion of the two hormones that control ovulation, luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Senger, 2003). Immunocontraceptive vaccines combine GnRH conjugated with a larger protein and an adjuvant. Antibodies are generated which bind GnRH to prevent stimulation of the pituitary gland and consequently suppress LH and FSH secretion.

Killian et al. (2004) showed immunocontraceptives targeting GnRH to be an effective method of contraception and somewhat effective long term. Fifteen mares treated with a single application of GonaCon™ GnRH immunocontraceptive were monitored over a four year period for contraception and antibody titer levels. Antibody levels declined over time, and contraception rates while exposed to a fertile stallion were

94%, 60%, 60% and 40% respectively over the four years. Although GnRH vaccines show promise, Miller et al. (2008) believes that current GnRH vaccines could not be considered highly effective long term. Zona pellucida based immunocontraceptives show promise to have the safety and remote delivery advantages of GnRH vaccinations, but with higher and longer lasting efficacy (Killian et al., 2004).

The Zona Pellucida as an Immunocontraceptive Target

The zona pellucida (ZP) is an acellular glycoprotein matrix that forms between the membrana granulosa and the oocyte during the second stage of folliculogenesis (Senger, 2003). It provides protection for the oocyte and early embryo, is involved with sperm recognition, binding and penetration, prevents polyspermy, and initiates the acrosome reaction (Wassarman, 2008). The ZP of most mammalian species is comprised of three glycoproteins, termed ZP1, ZP2, and ZP3 (Wassarman, 2008). ZP3 is responsible for sperm recognition and binding, and induction of the acrosome reaction (Wassarman, 2008). Porcine ZP has small amounts of ZP2, but the pZP3 family contains two immunologically and structurally distinct glycoproteins, ZP3α and ZP3β (Sacco, 1989). Sacco et al. (1989) showed that pZP3α is involved in sperm binding, but ZP3β is not.

The ZP is an ideal target for immunocontraception because it is involved in sperm binding, and is therefore essential for fertilization. Injection of adjuvanted porcine ZP3 (pZP) will generate an active immune response against the female's own ZP3, and cause contraception (Barber and Fayer-Hoskens, 2000a). Porcine ZP immunocontraceptive

shows promise as a safe and feasible fertility control method for feral horses. Risk of toxic side effects is low because the ZP is specific to the ovary, meaning anti-ZP antibodies are unlikely to react with somatic tissue in other parts of the body (Barber and Fayer-Hosken, 2000b). Barber and Fayer-Hoskens (2000b) also showed that adjuvanted pZP is not toxic to non-target animals when ingested orally, and does not influence their reproductive abilities. Because antigenic determinants on the ZP are shared across species, an immune response can be generated in horses using pZP, which can be obtained inexpensively and in large quantities (Yurewicz et al., 1987).

Vaccination with pZP3 has been shown in multiple studies to cause the mare to generate antibodies against the ZP and cause contraception. Liu et al. (1989) performed the first trial of pZP vaccination in horses. Fourteen fertile mares were given four injections of heat solubilized pZP at two to four week intervals and a booster six to ten months later. Aluminum hydroxide gel was initially used as an adjuvant, and replaced with Freund's adjuvant due to low efficacy. Contraception for at least 7 months was seen in 86% of mares.

Kirkpatrick et al. (1990; 1991; 1992) studied pZP vaccination using feral mares on Maryland's Assateague Island. They gave an initial injection of pZP with Freund's Complete Adjuvant (FCA), followed by two booster injections of pZP with Freund's Incomplete Adjuvant (FIA). Two annual booster vaccinations were given and foaling rates of treated, control and untreated mares were monitored over the three years following the initial injection. The first year, a 50% decrease in foaling was reported, the second year foaling rate decreased to 7%, compared to 44% and 50% in control and untreated mares, and by the third year 100% contraception was observed (Kirkpatrick et

al., 1990; 1991; 1992). This study also revealed that pZP had no harmful effects on existing pregnancies, however a few mares did develop injection site reactions (Kirkpatrick et al., 1990). Willis et al. (1994) showed that remote delivery is possible with pZP vaccines by administering an initial vaccine and a booster of a pZP formulation with a biobullet to six mares. Contraception for two years was observed in all six mares (Willis et al. (1994).

To date the only pZP preparation that can provide long term contraception from a single dose is SpayVac™ (SpayVac). SpayVac utilizes liposome encapsulation of ZP antigens to provide a boosting effect after the injection (Maudlin et al., 2007). Killian et al. (2008) tested SpayVac on a group of 12 mares. After a single injection of SpayVac, contraception rates of 100%, 83%, 83% and 83% were observed over the next four years, respectively (Killian et al., 2008).

In Vitro Methods for Evaluating Efficacy

Liu et al. (1989) used two in vitro methods for evaluating the efficacy of the pZP vaccine tested. An indirect immunofluorescence assay was used to determine the ability of anti-ZP antibodies to bind to the ZP of equine and porcine oocytes. Oocytes were collected, and incubated first in antiserum from mares inoculated with pZP vaccine, then in fluorescein isothiocyanate (FITC)-conjugated rabbit anti-horse IgG diluted 1:10 (Liu et al., 1989). Equine anti-serum was shown to bind with the ZP of equine and porcine oocytes as determined by quantifying fluorescence using an Olympus B2-2 microscope.

Liu et al. (1989) used sperm binding inhibition assays as a second method for in vitro assessment. Equine, porcine, and bovine oocytes were incubated in equine anti-pZP serum, and washed three times by centrifugation. Treated oocytes were incubated overnight in capacitated equine or porcine sperm, and the number of sperm tightly bound to each oocyte was counted. Sperm binding inhibition was observed in porcine and bovine oocytes incubated with pZP anti-sera as compared to oocytes incubated with control sera, although results were not obtained for sperm binding to horse oocytes due to inconsistent controls. The results of these two assays are consistent with the high contraceptive rates seen in mares immunized with the pZP preparation.

VanderVoort (1995) also used a sperm binding inhibition assay to test the efficacy of various recombinant ZP proteins as immunocontraceptives in monkeys. Oocytes were incubated with control sera, or anti-sera against various ZP preparations for either four or 24 hours. After incubation of treated oocytes with sperm, the number of sperm bound to each oocyte was counted. Significant differences in sperm binding between the control oocytes, and those treated with a preparation of the 55 kD ZP protein, were seen for both four and 24 hour treatment incubation groups. VanderVoort (1995) used this information in his conclusion that the 55 kD ZP protein serves as the sperm receptor.

Bartell (2011) evaluated the efficacy of the pZP preparation SpayVac as an immunocontraceptive in horses using both an indirect immunofluorescence assay with bovine oocytes and a sperm binding inhibition assay with bovine oocytes and sperm. To evaluate anti-pZP binding, oocytes were incubated in Dulbecco's phosphate buffered saline (DPBS), or 0.05% pooled sera from control, pre-immune, or high anti-pZP titer mares, washed, and incubated with FITC-conjugated rabbit anti-horse IgG at a

concentration of 1:64 for one hour. Oocytes treated with high titer anti-pZP sera fluoresced with significantly higher intensity than oocytes treated with DPBS or pre-immune sera, however no significant difference in fluorescence was observed between oocytes treated with control and high titer anti-pZP sera.

Bartell (2011) also evaluated inhibition of bovine sperm binding to bovine oocyte ZP by anti-pZP sera. Inhibition of sperm binding was evaluated by treating bovine oocytes with DPBS, or 0.05% pooled sera from control, pre-immune, or high anti-pZP titer mares for two hours, washing oocytes, then incubating oocytes with bovine sperm for 4 hours. The number of sperm tightly bound to each oocyte was counted. Number of sperm bound to oocytes treated with high titer anti-pZP serum was significantly lower than the number of sperm bound to oocytes treated with pre-immune serum or DPBS, but not significantly different from the number of sperm bound to oocytes treated with control serum.

In both of the assays used by Bartell (2011), statistically significant differences between high titer anti-pZP and control sera could not be detected. Sera collected from control mares have no pZP titers so a precise in vitro assay should be able to confidently discriminate between high titer anti-pZP and control sera. Therefore, the objective of this research was to refine the protocols used by Bartell (2011) to develop more precise assay conditions where obvious differences between high titer anti-pZP and control sera could be detected with statistical significance.

MATERIALS AND METHODS

Indirect Immunofluorescence Assay

Bovine ovaries were obtained from a local abattoir, frozen and thawed to room temperature before use. To collect oocytes, ovaries were ground gently in a tissue homogenizer with DPBS. The resulting liquid and ground tissue were strained through a gauze filter and a Teflon screen. This grinding and filtration process was repeated three times, so that for the final filtration, the ovarian tissue was completely homogenized. The Teflon screen was inverted over a collection dish, and oocytes were recovered from the surface by rinsing thoroughly with a jetted stream of DPBS. The collection dish was searched using a dissecting microscope at 20X magnification, and oocytes without cumulus cells were removed into DPBS with 0.5% bovine serum albumin (BSA).

Recovered oocytes were fixed in a 2% paraformaldehyde in DPBS solution for at least one hour and washed through three 50 µL microdrops of DPBS with 0.5% BSA under paraffin oil. Oocytes were blocked for one hour in 50 µL microdrops of DPBS with 5% heat treated fetal calf serum (HTFCS), and then washed through three microdrops of DPBS with 0.5% BSA. Blocked oocytes were separated into 5 treatment groups, and treated for 1 hour in 50 µL microdrops of DBPS, or DPBS with 1:200 dilutions of pooled sera from pre-immune, control, or low or high pZP titer mares vaccinated with SpayVac. Oocytes were washed through three microdrops of DPBS with 0.5% BSA, incubated for 1 hour in FITC-conjugated rabbit anti-horse IgG at dilutions of 1:32, 1:40, 1:64 and 1:128. and washed again through three microdrops of DPBS and

0.5% BSA. Fluorescence intensity was evaluated using epifluorescence on a Leitz inverted stage phase contrast microscope with a FITC filter and quantified using a Leitz photometer.

The DPBS group was not repeated in trials with 1:40 and 1:128 concentrations of FITC-conjugated rabbit anti-horse IgG, as the trials with 1:32 and 1:64 dilutions were sufficient to show significantly low anti-pZP binding. The low titer group was not used in the trial with the 1:128 dilution of FITC-conjugated rabbit anti-horse IgG, as the first three trials were sufficient to show a significant difference in anti-pZP binding between low and high titer groups.

Sperm Binding Inhibition Assay

Bovine oocytes were collected using the process described above. Recovered oocytes were vortexed for 15 seconds at high speed to remove cumulus cells. Oocytes were washed through three 50 µL microdrops of DBPS with 0.5% BSA, fixed for two hours in 2% paraformaldehyde, and washed through three microdrops of Sperm TALP (Parrish et al., 1988), Oocytes were incubated for two hours in one of five treatments: DPBS, or 1:200 dilutions of pooled sera from pre-immune, control low or high titer anti-pZP sera from mares incubated with SpayVac. Oocytes were washed through four microdrops of sperm TALP. Frozen bovine semen in a 0.5 cc straw was thawed in a water bath at 37°C for 45 seconds. Motile sperm were selected using the sperm swim-up procedure described by Parrish et al. (1988) and suspended in Sperm TALP. Oocytes were added to 40 µL microdrops of Sperm TALP containing 25,000 sperm, and

incubated overnight at 39°C in a humidified atmosphere of 5% CO_2 in air. Sperm and oocytes were placed in a microcentrifuge tube with 100 μL of DPBS-BSA and vortexed at a low fixed speed for 15 seconds to remove loosely bound sperm from oocytes. Sperm-bound oocytes were recovered from the microcentrifuge tube, washed through three drops of DPBS-BSA, and fixed in paraformaldehyde for at least one hour. Fixed oocytes were washed through four microdrops of DPBS-BSA, and incubated for 10 min in the dark with 1% Hoechst 33324 in 25% ethanol in sodium citrate dehydrate solution. Oocytes were washed through three microdrops of DPBS-BSA, and the number of sperm tightly bound to each oocyte was counted using epifluorescence on a Leitz inverted stage-phase contrast microscope at 200X magnification.

RESULTS

Indirect Immunofluorescence Assay

Fluorescence intensity was higher ($P < 0.05$) for oocytes treated with high compared to low titer anti-pZP sera when 1:32, 1:40, and 1:64 dilutions of FITC-conjugated rabbit anti-horse IgG were used (Figures 1 and 2). Oocytes treated with sera from mares vaccinated with SpayVac fluoresced significantly more ($P < 0.05$) intensely than oocytes treated with sera from control or pre-immune mares, or DPBS, for all concentrations of FITC-conjugated rabbit anti-horse IgG (Figures 1, 2, and 3).

Oocytes treated with the 1:32 dilution of FITC-conjugated rabbit anti-horse IgG fluoresced more ($P<0.05$) intensely than oocytes treated with all lower concentrations. No significant difference was observed between oocytes treated with 1:40 and 1:64 dilutions of FITC-conjugated rabbit anti-horse IgG. Oocytes treated with a 1:128 dilution of FITC-conjugated rabbit anti-horse IgG fluoresced less ($P<0.05$) intensely than oocytes treated with all higher concentrations.

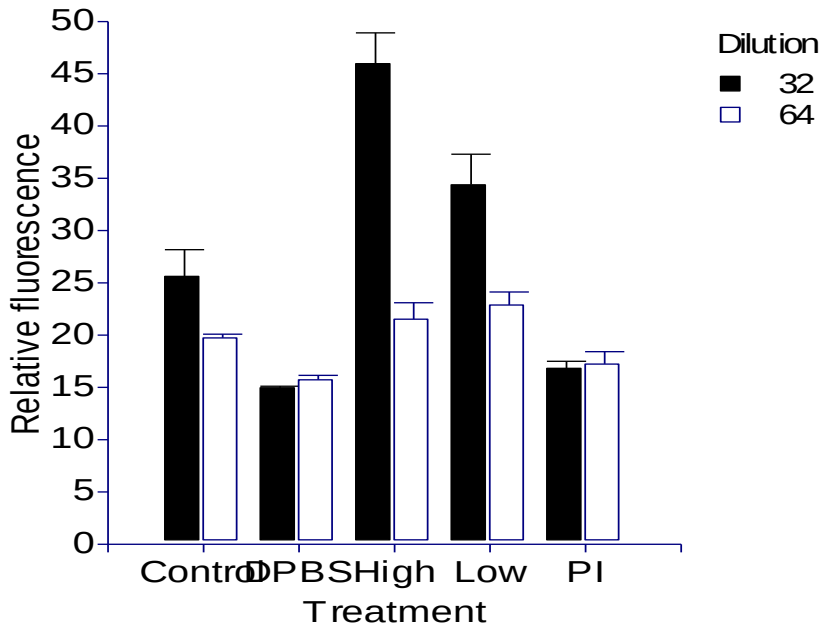

Figure 1. Mean relative fluorescence for bovine oocytes incubated in DPBS or pooled sera recovered from pre-immune, control, low or high pZP titer mares and exposed to rabbit anti-horse IgG-FITC conjugate at 1:32 or 1:64 dilutions.

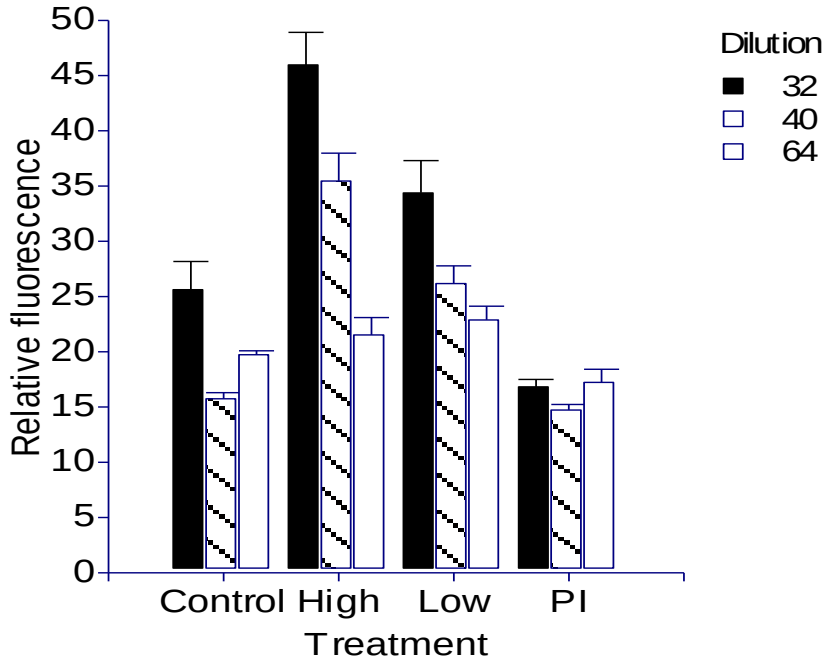

Figure 2. Mean relative fluorescence for bovine oocytes incubated in pooled sera recovered from pre-immune, control, low or high pZP titer mares and exposed to rabbit anti-horse IgG-FITC conjugate at 1:32, 1:40, or 1:64 dilutions.

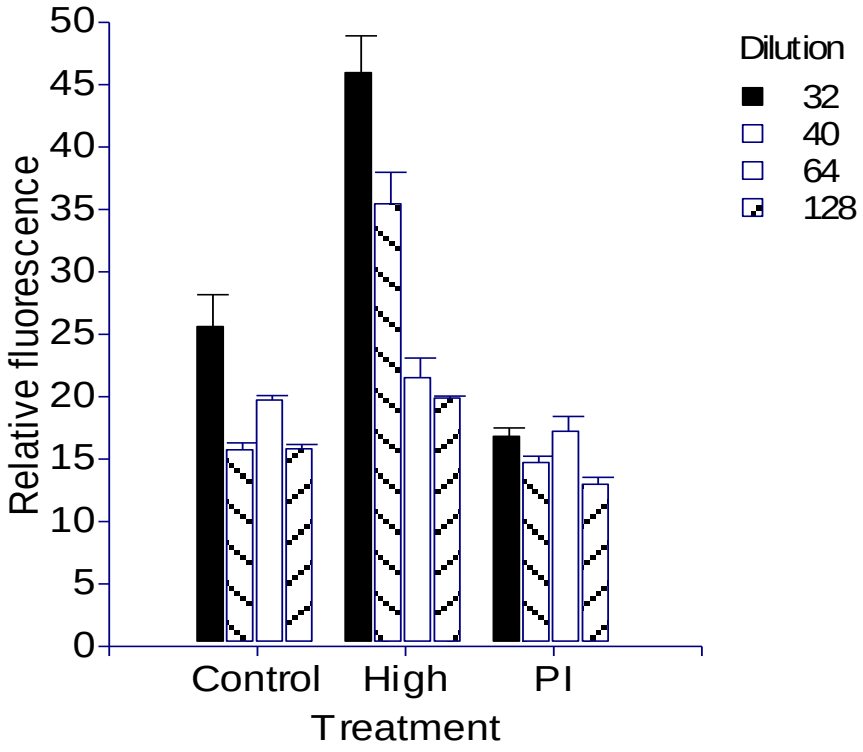

Figure 3. Mean relative fluorescence for bovine oocytes incubated in pooled sera recovered from pre-immune, control or high pZP titer mares and exposed to rabbit anti-horse IgG-FITC conjugate at 1:32, 1:40, 1:64, or 1:128 dilutions

Sperm Binding Inhibition Assay

Sperm binding was lower (P<0.05) on oocytes treated with anti-pZP sera from mares treated with SpayVac than sera from pre-immune mares (Figure 4). No significant difference was observed between oocytes treated with DPBS, control or high titer sera, or between oocytes treated with DPBS, control or pre-immune sera, although a higher mean number of sperm bound was observed on oocytes treated with DPBS and control sera than high titer sera.

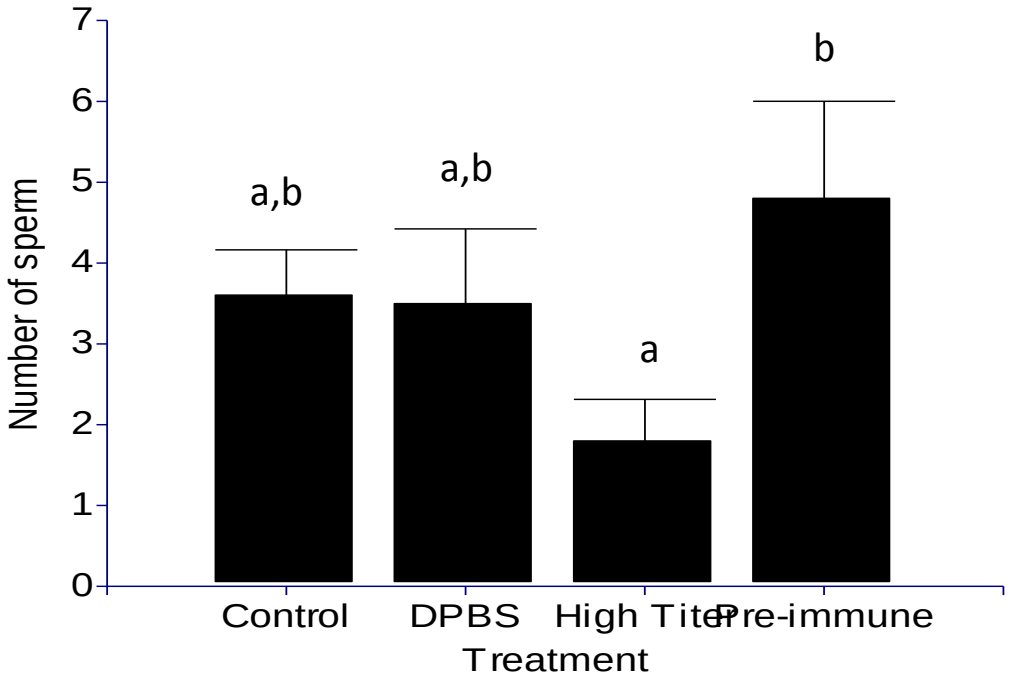

Figure 4. Mean number of bovine sperm bound to bovine oocytes following incubation in DPBS or pooled sera recovered from pre-immune, control, or high pZP titer mares.

a,b Means without common superscripts differ (P<0.05).

DISCUSSION

Indirect Immunofluorescence Assays

Bartell (2011) showed that indirect immunofluorescence assays will confirm anti-pZP binding in bovine oocytes, but significant differences in fluorescence intensity were only observed between anti-pZP serum treated oocytes, and pre-immune serum and DPBS treated oocytes. In this study, significantly higher florescence intensity was observed in oocytes treated with anti-pZP serum compared to oocytes treated with DPBS, control serum or pre-immune serum. Florescence intensity was also shown to reflect the titer level of anti-pZP sera used. Ocytes treated with high titer anti-pZP serum fluoresced at a higher intensity than oocytes treated with low titer anti-pZP serum. It was also shown that florescence intensity increases with higher concentrations of rabbit anti-horse FITC. Rabbit anti-horse FITC concentration of 1:32 produced results with the most distinction between florescence intensity of oocytes treated with control, and high and low titer anti-pZP sera.

Sperm Binding Inhibition Assay

Bartell (2011) reported significantly lower sperm binding in oocytes treated with high titer anti-pZP serum than oocytes treated with pre-immune serum and DPBS, but did not find a significant difference between oocytes treated with high titer anti-pZP serum and control serum. It was hypothesized that by extending the incubation of oocytes with

sperm from four hours to overnight, results could be obtained that showed a significant difference between oocytes treated with high titer anti-pZP serum and control serum. Although the difference between high titer serum, and pre-immune serum and DPBS seen in Bartell (2011) was replicated in this study, no significant difference was seen between high titer and control sera. Extending the sperm-oocyte incubation period did not improve precision in the sperm binding assay.

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